

# The Human E48 Antigen, Highly Homologous to the Murine Ly-6 Antigen ThB, Is a GPI-anchored Molecule Apparently Involved in Keratinocyte Cell-Cell Adhesion

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**Abstract.** The E48 antigen, a putative human homologue of the 20-kD protein present in desmosomal preparations of bovine muzzle, and formerly called desmoglein III (dg4), is a promising target antigen for antibody-based therapy of squamous cell carcinoma in man. To anticipate the effect of high antibody dose treatment, and to evaluate the possible biological involvement of the antigen in carcinogenesis, we set out to molecularly characterize the antigen. A cDNA clone encoding the E48 antigen was isolated by expression cloning in COS cells. Sequence analysis revealed that the clone contained an open reading frame of 128 amino acids, encoding a core protein of 13,286 kD. Database searching showed that the E48 antigen has a high level of sequence similarity with the mouse ThB antigen, a member of the Ly-6 antigen family. Phos-

phatidylinositol-specific (PI-specific) phospholipase-C treatment indicated that the E48 antigen is glycosylphosphatidylinositol-anchored (GPI-anchored) to the plasma membrane. The gene encoding the E48 antigen is a single copy gene, located on human chromosome 8 in the 8q24-qter region. The expression of the gene is confined to keratinocytes and squamous tumor cells. The putative mouse homologue, the ThB antigen, originally identified as an antigen on cells of the lymphocyte lineage, was shown to be highly expressed in squamous mouse epithelia. Moreover, the ThB expression level is in keratinocytes, in contrast to that in lymphocytes, not mouse strain related. Transfection of mouse SV40-polyoma transformed mouse NIH/3T3 cells with the E48 cDNA confirmed that the antigen is likely to be involved in cell-cell adhesion.

THE monoclonal antibody E48 (mAb E48) has been developed for the eradication of minimal residual disease in patients with squamous cell carcinoma of the upper aerodigestive tract. The antibody has been tested extensively in in vivo model systems as well as in radioimmunoscintigraphy studies in patients with squamous cell carcinoma. Biodistribution and dosimetry calculations indicated the feasibility of adjuvant radioimmunotherapy for the eradication of minimal residual disease after initial surgical treatment (van Dongen et al., 1994; de Bree et al., 1994, 1995).

The mAb E48 defined target antigen is a small membrane protein (15–22 kD) exclusively expressed at the outer cell surface of squamous carcinoma cells and their normal counterparts: the cells of transitional epithelia and the keratinocyte of stratified squamous epithelia (Quak et

al., 1990). In vitro experiments provided evidence that the antigen is involved in the (desmosomal) cell-cell adhesion of keratinocytes (Schrijvers et al., 1991).

Various filament anchorage systems of cellular adhesion exist in a particular type of epithelial cell. Among the adhering junctions which require calcium ions for functioning, two major categories can be distinguished. One category consists of junctions attached to the microfilaments of the cytoskeleton (actin). These junctions are composed of a transmembrane complex, containing adhesion molecules of the cadherin family, and a plaque structure formed by the cytoplasmic domain of the cadherins and a complex set of associated proteins. In the E-cadherin complex, the actual adhesion molecule E-cadherin, a 120–140-kD transmembrane protein, is associated with plakoglobin, a structural component of all calcium-dependent junctions, and the  $\alpha$ - and  $\beta$ -catenins ( $\gamma$ -catenin is thought to be identical to plakoglobin) which are thought to be involved in the structural and functional interactions with for instance the cytoskeleton (Takeichi, 1990; Geiger and Ayalon, 1992).

Besides their function in cell adhesion, the junctions are involved in signal transduction processes implicated in al-

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terations of cell density, loss of matrix adherence etc., and can in this way influence cell behavior and morphology (Vleminckx et al., 1991; Wheelock and Jensen, 1992; Schmidt et al., 1993). The interaction of protein kinase C and protein kinases of the src family with E-cadherin adhesion junctions, has been established by several lines of evidence, and these molecules may well be involved in the signal transduction pathways (Tsukita et al., 1991; Geiger and Ayalon, 1992). Moreover, the role of E-cadherin junctions in cellular growth has been determined in vitro as well as in vivo, and one of the proteins associated with the junctions,  $\beta$ -catenin, interacts with the gene product of the APC tumor suppressor gene, a gene in which loss of function mutations are involved in the development of epithelial tumors (Su et al., 1993).

The second category of cellular junctions consists of desmosomes and hemidesmosomes, the attachment sites for the intermediate filaments: the cytokeratins in epithelial cells and desmin and vimentin in most other cells. The desmosomes contain the cadherins desmocollin and desmoglein, which form the actual cellular connection, as well as the plaque proteins plakoglobin, desmoplakin, and several other cell-type specific components (Schwarz et al., 1990; Royanovsky et al., 1993). The involvement of these second category junctions on the behavior of the cell is less well defined. Keratinocytes of squamous epithelia are attached to their adjacent cells by large numbers of desmosomes, and it seems obvious that these cellular organelles may play important roles in the development of transformed cells to invading and metastatic tumor cells, but knowledge of this kind is still scarce.

Although a large number of the proteins present in bovine muzzle desmosome preparations have been identified and the genes encoding them have been cloned, some of the components, such as the small 20-kD glycoprotein are still uncharacterized. Formerly this glycoprotein was termed desmoglein III or dg 4, but the term desmoglein has recently been reserved for a subgroup of the desmosomal cadherins (Gorbsky and Steinberg, 1981; Schwarz et al., 1990; Buxton et al., 1993). The restricted pattern of expression and additional experimental data suggested that the mAb E48 defined antigen is identical to that 20-kD glycoprotein. Immunoelectronmicroscopical analysis with mAb E48 on squamous tissue sections showed that a considerable amount of antigen is located in the midline of the desmosomes, indicating a putative involvement in desmosome formation. Moreover, squamous carcinoma cells seeded within collagen gels grow as colonies, whereas after treatment of the cells with mAb E48, the colony phenotype changes to a dispersed phenotype, indicating that the binding of the antibody inhibited cell-cell adhesion (Schrijvers et al., 1991). Together these data strongly support the hypothesis that the human E48 antigen is identical to the 20-kD glycoprotein detectable in bovine desmosomal preparations.

To unravel the involvement of the membrane protein E48 in the adhesion of epithelial cells, we set out for the molecular cloning of the cDNA encoding the E48 antigen, and evaluated the effect of the antigen in cells which normally do not express it. In this paper we report on the interesting features of the molecule, and its apparent role in keratinocytes.

## Materials and Methods

### Materials

Restriction enzymes were purchased from Boehringer Mannheim (Almere, The Netherlands) or Pharmacia (Pharmacia Biotech Benelux, Roosendaal, The Netherlands). DNA polymerase (Klenow fragment) and T4 DNA ligase were from Boehringer Mannheim. AMV reverse transcriptase was from Promega (Leiden, The Netherlands), and T4 polynucleotide kinase as well as T7 DNA polymerase used for sequencing were from Pharmacia. The reaction conditions were chosen as recommended by the supplier or according to Sambrook et al. (1989).  $^{32}$ P-labeled nucleoside triphosphates (3,000 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA). The expression vector pCDM8 was obtained from Invitrogen (Sanbio, Uden, The Netherlands), and the CD2 cDNA clone in pCDM8 was kindly provided by Dr. B. Seed (Seed and Aruffo, 1987). The cDNA clone encoding the ThB antigen was kindly provided by Dr. M. Sandrin (Gumley et al., 1992). Kits for sequence analysis on automated systems were obtained from Applied Biosystems (Maarsse, The Netherlands).

### Cell Lines and Tissue Culture

The HNSCC (Squamous Cell Carcinoma of the Head and Neck) cell lines UM-SCC-22A and UM-SCC-22B, used for Northern blotting and cDNA synthesis (UM-SCC-22A) were kindly provided by Dr. T. E. Carey (Ann Arbor, MI). The cell lines OVCAR-3, WiDr-29, and A-431 were obtained from Dr. H. Haisma, LNCAP from Dr. A. A. Geldof, Tera-1 and MCF-7 from Dr. G. Giaccone, DOHH2 from Dr. J. W. van Oostveen, NB-1 from Dr. A. J. Langeveld, U-715 and Raji from Dr. A. Dräger, Hela and SiHa from Dr. C. J. L. M. Meijer. KB cells from Dr. G. Jansen (all from the Free University, Amsterdam, The Netherlands), and PC112 from Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, The Netherlands). The tissue origin of forementioned human cell lines is indicated in Table I. COS-7 cells were obtained from Dr. D. H. Joziasse (Free University, Amsterdam) and MOP-8 cells (SV40-polyoma-transformed NIH/3T3 cells) from the American Tissue Culture Collection (Rockville, MD).

Cells were routinely cultured in humidified air/5% CO<sub>2</sub> at 37°C in DMEM (Gibco Life Technologies, Breda, The Netherlands), 5% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, 1% penicillin/streptomycin (ICN Biomedicals B.V., Amsterdam, The Netherlands), 16 mM NaHCO<sub>3</sub> and 15 mM Hepes, pH 7.4, or RPMI 1640 (ICN Biomedicals, B.V.), 5% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin.

Human primary keratinocytes were isolated from Uvulo-Palato-Pharyngeal surgical specimens as described for foreskin keratinocytes (Bruynzeel et al., 1990). The cells were cultured in Gibco Keratinocyte Growth Medium (KGM) according to the manufacturer's instructions. Mouse epidermis was isolated from tail skin by a similar thermolysin treatment as described for the isolation of human keratinocytes.

As checked by immunocytochemistry of cytospin preparations, 100% of the UM-SCC-22A cells, 20% of the UM-SCC-22B cells, and none of the COS-7 as well as MOP-8 cells expressed the E48 antigen, irrespective of the culture conditions.

### SDS-PAGE and Immunoblotting

UM-SCC-22B, UM-SCC-22A cells cultured in monolayer to 80% confluency, and transfected COS-7 cells were washed with PBS, detached by trypsinization and lysed in Laemmli sample buffer without  $\beta$ -mercaptoethanol (Laemmli, 1970). Lysates were boiled, centrifuged, and applied on a miniprotein II system (BioRad Labs., Hercules, CA) for SDS-PAGE performed as described by Laemmli, using a 12.5% slab gel. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose (Schleicher and Schüll, Dassel, Germany) or PVDF was performed by a mini-transblot electrophoretic cell (Bio-Rad), at 100 V for 1 h (Sambrook et al., 1989). After transfer, the nitrocellulose membrane was cut into strips and incubated at room temperature with 2% BSA (Fraction V) in PBS to block free binding sites on the membrane. The "blocked" nitrocellulose membrane strips were incubated for 1 h with  $^{125}$ I-labeled mAb E48 (100,000 cpm/ml). Finally, nitrocellulose strips were washed 3  $\times$  10 min with PBS/0.05% Tween-20, dried, and autoradiographed by exposure to Kodak X-AR 5 (Kodak, Odijk, The Netherlands) film for 1-7 d at -70°C.

### Isolation of Desmosomes

The xenograft line HNX-22B was used as source for the isolation of hu-

man desmosomes. The establishment and maintenance of the xenograft line HNX-22B by serial transplantation was performed as described earlier (Schrijvers et al., 1991). Desmosomes were isolated essentially as described by Gorbisky and Steinberg (1981), Franke et al. (1981), and Skerrow et al. (1987), except that reducing agents were left out of the buffers as the mAb E48 defined epitope is sensitive to reduction. In short: 3 g of human xenograft material was minced by scissors and suspended in 100 ml CASC-A buffer containing 0.1 M citric acid, pH 2.3, 0.05% NP-40, 5 µg/ml pepstatin, 5 µg/ml leupeptin, 0.05 mM PMSF. The suspension was stirred vigorously for 30 min at 4°C, homogenized in a Polytron homogenizer (five times 30 s with 1 min interval) and centrifuged for 20 min at 13,000 g. The pellet was resuspended by vortexing in 12 ml CASC-B buffer containing 0.1 M citric acid, pH 2.3, 0.01% NP-40, 5 µg/ml pepstatin, 5 µg/ml leupeptin, 0.05 mM PMSF, and the large debris was removed by centrifugation for 20 min at 750 g. The desmosomal fraction was collected by centrifugation for 20 min at 10,000 g. The pellet was homogenized by a Dounce homogenizer. This procedure, centrifugation and homogenization, was repeated twice. The last time the pellet was resuspended in 1.2 ml 40% sucrose in CASC-B buffer and loaded on a discontinuous gradient consisting of 1.2 ml 50%, 1.2 ml 55%, and 1.2 ml 60% sucrose in CASC-B buffer in a 5-ml polyallomer tube, followed by centrifugation at 180,000 g for 3 h at 10°C. The material concentrated at the 55–60% sucrose interface was collected by aspiration, diluted with CASC-B buffer to 12 ml and centrifuged at 22,000 g for 20 min at 4°C. This step was repeated once, and the final pellet was resuspended in 1 ml CASC-B. About 2 µg protein was run on a 12.5% SDS-PAGE gel under nonreducing conditions and the gel was either stained by Coomassie brilliant blue, or used for immunoblotting as described below.

### Antigen Isolation

Alternatively, for preparative antigen isolation, HNX-22B xenografts were cut into small pieces and snap frozen in liquid nitrogen. The frozen material was pulverized in a micro-dismembrator (Braun AG, Melsungen, Germany) and subsequently resuspended in cold extraction buffer (EXB: 20 mM TRIS.HCl, 150 mM NaCl, 1% [wt/vol] *n*-octyl-β-D-glucoside, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml trypsin inhibitor, 1 mM PMSF, pH 8.0; 3 ml EXB/mg pulverized tissue). All further steps were performed on ice in the presence of forementioned protease inhibitors. The suspension was sonicated three times for 10 s at 10,000 Hz, and centrifuged at 4°C for 1 h at 100,000 g to obtain a clear supernatant. The proteins were precipitated from the clear supernatant by adding 10 vol precooled acetone-methanol (1:1). The mixture was allowed to stand for 4 h with occasional swirling. The precipitated proteins were collected by centrifugation at 100,000 g for 1 h at 4°C, the pellet resuspended in precooled ethylether and the precipitated proteins recollected by centrifugation at 4°C for 1 h at 100,000 g. After evaporation of the solvent, the proteins were dissolved in the original volume of EXB. After centrifugation of the solution at 100,000 g for 1 h at 4°C, the E48 antigen was isolated by immunoabsorption. Purified mAb E48 IgG (kindly provided by Centocor Europe Inc., Leiden, The Netherlands), was coupled to Affigel-10 (BioRad Laboratories B.V., Veenendaal, The Netherlands) in 0.1 M MES (2-[*N*-morpholino]ethanesulfonic acid), pH 4.8 for 16 h at 4°C. Unbound IgG was removed by washing the material twice with MES for 10 min and twice with EXB. The redissolved protein extract was incubated with the immunoabsorbent for 16 h at 4°C under continuous gentle agitation, and washed with EXB three times. The immunoabsorbed material was eluted in 3 × 1 ml 50 mM triethanolamine pH 12.5, 1% *n*-octyl-β-D-glucoside. The eluted fractions containing antigen were concentrated with Centricon-3 microconcentrators, mol wt cut-off 3,000 Dalton (Grace B. V. Amicon Division, Rotterdam, The Netherlands). Approximately 2 µg protein was run on an SDS-PAGE gel under nonreducing conditions, blotted to Problot™ PVDF (polyvinylidene difluoride, Applied Biosystems). After transfer, the proteins on the membrane were stained with 0.1% Amido Black in 1% acetic acid, 40% methanol. The protein band of 20 kD was cut out and the amino acid sequence determined by a protein sequencer (Applied Biosystems, model 473A). The isolation was followed by immunoblotting of samples taken in every step. Small aliquots of the purified antigen were radioiodinated and analyzed for purity.

### Phosphatidylinositol-Specific Phospholipase C-Treatment and FACS Analysis

5 × 10<sup>6</sup> UM-SCC-22A and various control cells were washed with anion-free buffer (225 mM sucrose, 20 mM Hepes, pH adjusted to 7.4 with solid MgO and filtered (Henderson and Zevely, 1980), and treated with 0.3 U

phosphatidylinositol (PI)-specific phospholipase-C (Boehringer Mannheim) in 200 µl anion-free buffer at 37°C for 1 h. Cells were subsequently spun on microscopical glass slides, and stained by immunocytochemistry, or washed with PBS (Sambrook et al., 1989) and prepared for immunoblotting or FACS (fluorescence-activated cell sorting) analysis. For FACS analysis, the treated cells were incubated with the optimal concentration of mAbs in ice-cold PBS/0.1% BSA for 45 min at 0°C. After washing in ice-cold PBS/0.1% BSA, the cells were incubated with fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin conjugate (Becton-Dickinson, Eten-Leur, The Netherlands) for 30 min at 0°C in the dark. After washing the cells were analyzed immediately by a FAC-Scan (Becton-Dickinson). The assay was checked by the incubation of KB cells, overexpressing the glycosyl-phosphatidylinositol (GPI)-anchored folate receptor, and the anti-folate receptor antibody MOv18, and various antibody controls (Luhers and Slomiany, 1989; Coney et al., 1991).

### Probes

DNA fragments were separated from plasmid sequences by "Freeze and Squeeze". In short, DNA digests were run on an NA agarose gel (Pharmacia), the bands cut out of the gel and frozen. The frozen blocks were put between parafilm and the solution squeezed out (usually with a glass plate), followed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation (Sambrook et al., 1989). Probes were made by labeling the isolated inserts with [ $\alpha$ -<sup>32</sup>P]dCTP (New England Nuclear Research Products, 3,000 Ci/mmol) by means of multiprimed elongation (Feinberg and Vogelstein, 1983).

### DNA Isolation, Southern Blotting, and Hybridization

DNA was isolated from tissue culture cells according to Brakenhoff et al. (1990). 10–15 µg of DNA was digested to completion with restriction enzymes, loaded on a 0.7% agarose gel and electrophoresed in TAE buffer according to Sambrook et al. (1989). The DNA was denatured by soaking the gel in 0.4 M NaOH, 0.6 M NaCl for 30 min, and blotted by capillary transfer to genescreen-plus filters (New England Nuclear Research Products) in the same solution. After transfer the blot was neutralized in 1 M NH<sub>4</sub>Acetate/0.02 M NaOH for 5 min and washed in 2× SSC. The filter was baked for 2 h at 80°C, prehybridized in 7% SDS, 0.5 M sodium phosphate buffer, 2 mM EDTA, pH 7.0 for 2 h at 65°C, and after addition of the denatured probe hybridized at 65°C for 16 h. Filters were washed twice with 2× SSC, 0.2% SDS and twice with 0.2× SSC, 0.2% SDS at 65°C for 15 min, and the bands visualized by autoradiography with Kodak X-AR 5 film using intensifying screens.

### RNA Isolation and Northern Blotting

Total RNA was isolated from cultured cells according to Gough (1988), and from tissues using RNeasy (Cinna Biotecx: Campro Scientific, Veenendaal, The Netherlands). 20 µg of total RNA was loaded on a 1% agarose formaldehyde gel and electrophoresed in MOPS buffer essentially as described by Sambrook et al. (1989). The RNA was Northern blotted by capillary transfer in 10× SSC (Sambrook et al., 1989) onto genescreen plus filters, and hybridized as described above.

### Transfection and Immunocytochemistry

The DEAE-dextran transfection of COS-7 cells was essentially according to Brakenhoff et al. (1994b). COS-7 cells were trypsinized and plated 1–2 d before transfection in a 25-cm<sup>2</sup> culture flask (Greiner, Alphen a/d Rijn, The Netherlands). The next day cells were trypsinized, counted, and 10<sup>6</sup> cells resuspended in 0.5 ml RSC: RPMI 1640 (ICN Biomedicals B.V.), supplemented with 100 µM chloroquine (Promega) and 2% FCS (HyClone). 5–10 µg DNA (1 µg/µl) was added to 2 ml RSC and mixed with 2 ml RSC-DEAE: (800 µg/ml DEAE dextran (Promega) in RSC). After 2 min incubation at room temperature the cells resuspended in RSC were added, and the suspension was incubated for 2 h in the tissue culture incubator under 5% CO<sub>2</sub> at 37°C. The cells were subsequently spun at 800 g for 5 min and resuspended in 10 ml DMEM. 2 × 10<sup>6</sup> cells (2 ml) or 2 × 10<sup>4</sup> cells (200 µl) were added to 18 ml or 1.8 ml DMEM and seeded on a 70

1. Abbreviations used in this paper: CRG, cysteine-rich GPI-anchored; GPI, glycosyl-phosphatidylinositol; PI, phosphatidylinositol.

cm<sup>2</sup> or 9.6 cm<sup>2</sup> dish (Greiner, petridishes TC), respectively. After 48–72 h the cells were washed with PBS and air-dried.

The transfected cells were fixed for 10 min with ethanol 96%. The dishes were washed twice with PBS (5 min), preincubated for 15 min with 2% normal rabbit serum (NRS; Nordic Immunological Laboratories, Tilburg, The Netherlands) in PBS/1% BSA, and subsequently incubated for 1 h with an appropriate dilution of the specific mAb in PBS/1% BSA (2 ml per 70 cm<sup>2</sup> dish or 0.3 ml per 9.6 cm<sup>2</sup> dish, respectively). The dishes were washed 3× with PBS and subsequently incubated for 30 min with rabbit anti-mouse immunoglobulin (RAM; DAKO, Glostrup, Denmark) diluted 1:25 in PBS/1% NRS/1% BSA. The dishes were washed again three times with PBS and incubated for 1 h with alkaline phosphatase anti-alkaline phosphatase (APAAP; DAKO) diluted according to the supplier in PBS/1% BSA. The dishes were washed again three times with PBS, rinsed with alkaline phosphatase buffer (APB: 100 mM TRIS.HCl, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, pH 9.5) and incubated for 30 min with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (NBT, BCIP; both from Sigma-Aldrich N.V., Bornem, Belgium) in APB (5 µl NBT and 1 µl BCIP stock solution per ml APB; stock solutions: 20 mg/ml NBT in 70% dimethylformamide/30% demineralized water, and 50 mg/ml BCIP in dimethylformamide). Dishes were rinsed with demineralized water and the nuclei stained with nuclear fast red (Sigma) for 10 min (1 g nuclear fast red, 70 gram Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> per liter demineralized water, boiled in a waterbath and filtered before use). The dishes were rinsed again with demineralized water and evaluated under a microscope (Olympus, Paes Nederland B.V., Zoeterwoude, The Netherlands) at a magnification of 50× and 125×. The staining is preserved for at least one week without any loss of intensity.

MOP-8 cells, SV40-polyoma-transformed mouse NIH/3T3 cells, were transfected with DEAE dextran essentially as described for COS-7 cells. The amount of DEAE dextran, however, was reduced to 500 µg/ml. The amount of DNA decreased to 1 µg and the transfection time to 30 min.

### Construction of a UM-SCC-22A cDNA Library in pCDM8

Total RNA was isolated from UM-SCC-22A cells according to Gough (1988). 20 µg was primed with oligo-dT, and used for cDNA synthesis (Promega cDNA kit). The cDNA was ligated to noncomplementary EcoRI/BstXI adaptors (Invitrogen; Sanbio) and separated from nonligated adaptors on Sepharose CL 4B (Pharmacia) spin columns. The fractions containing cDNA larger than 500 bp were used for ligation into pCDM8 vector digested with BstXI (Seed 1987), and transformed to *E. coli* MC1061/P3 by electroporation as described by Rommens et al. (1991). The library,  $7 \times 10^5$  clones, was divided into nine fractions, grown overnight, frozen glycerol cultures prepared and DNA isolated by Qiagen tip-500 columns (Qiagen; Westburg, Leusden, The Netherlands) according to the supplier.

### Isolation of Clones by Screening Progressively Smaller Pools of Bacterial Colonies

The nine fractions of the library were transfected to COS-7 cells, and the cells immunocytochemically stained with mAb E48 as described. The fraction with the smallest number of bacterial colonies and the largest number of positively stained cells was identified, the frozen glycerol culture of that particular fraction titrated and the fraction divided into ten subfractions of 1/5 of the number of colonies from the parental fraction (thus screening a twofold excess of bacterial colonies). A sample of the inoculated culture was spread on agar plates to determine the exact number of colonies added. The subfractions were grown overnight, frozen glycerol cultures prepared and DNA isolated from the same culture, COS cells transfected and immunocytochemically stained. Repeated cycles of this analysis were performed until the cDNA clone of interest was enriched to homogeneity. DNA isolated by alkaline lysis (Sambrook et al., 1989) or Qiaprep-spin columns (Qiagen; Westburg) are all suited for transfection. To all DNA preparations a small amount (5%) of CD2 cDNA in pCDM8 was added to control the transfection efficiency of each particular sample by staining 10% of the transfected cells on a small dish with anti-CD2 antibody (Sanbio). Using this procedure of screening progressively smaller pools of bacterial colonies, the E48 antigen encoding cDNA could be isolated in six steps. More detailed descriptions of the methods are available upon request.

### Chromosomal Localization

Chromosomal assignment of the E48 antigen gene was performed using a

panel of well defined human-rodent somatic cell hybrids using the cDNA as a probe. Isolation of hybrid cell lines and determination of chromosome content has been described previously (Brakenhoff et al., 1990). Regional mapping was performed using fluorescence in situ hybridization essentially as described by Suijkerbuijk et al. (1992) with a genomic clone as a probe, and analyzed on a Zeiss Axiophot epifluorescence microscope. The clone was isolated from a genomic library of partial Sau3A.1 digested human DNA in EMBL-3 phages (Brakenhoff et al., 1994a).

## Results

### Presence of the E48 Antigen in Desmosome-enriched Fractions

The association of the E48 antigen with desmosomes has been established by several lines of evidence, but the presence of the antigen in desmosome-enriched preparations of human keratinocytes had not been determined (Schrijvers et al., 1991). Although this analysis was impeded by the fact that only limited material was available, we could show by immunoblotting that the E48 antigen is present in desmosome-enriched preparations (Fig. 1). This observation strongly supports the hypothesis that the E48 antigen is the human variant of the 20-kD glycoprotein present in desmosome-enriched preparations of bovine muzzle (Gorbsky and Steinberg, 1981; Franke et al., 1981; Skerrow et al., 1987; Schwarz et al., 1990).

### Isolation and Sequencing of the E48 Antigen

As a first step towards the molecular cloning of the E48 encoding cDNA and gene, the protein was isolated from

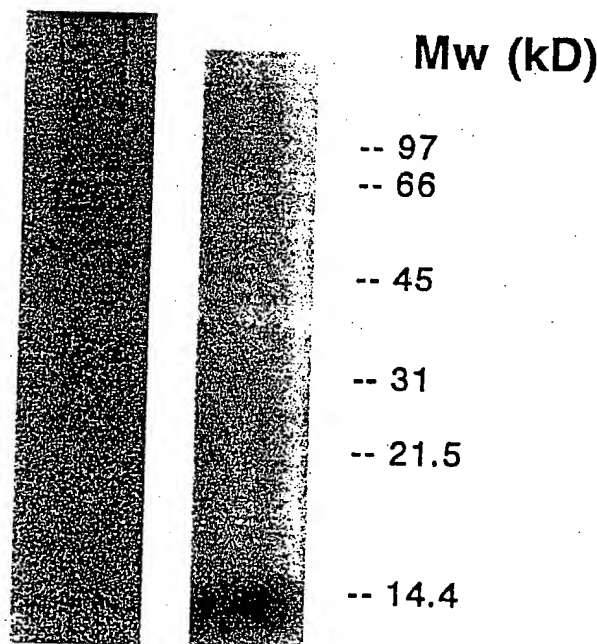


Figure 1. SDS-PAGE and immunoblotting of desmosome-enriched preparations. Desmosome-enriched fractions were prepared from human SCC xenografts grown in nude mice using citric acid extraction and sucrose gradient centrifugation in the absence of reducing agents. The material was run on an SDS-PAGE gel (also without reducing agent) and stained with Coomassie Brilliant Blue (left) or blotted, incubated with radio-labeled mAb E48, and exposed to Kodak X-AR 5 film for 16 h using intensifying screens (right).

xenograft extracts through affinity chromatography and SDS-PAGE. The isolation was hampered by the fact that the antigen was poorly soluble and tended to precipitate during the subsequent steps. Nevertheless, an isolation procedure could be established, enabling the purification of the E48 antigen in sufficient amounts for microsequencing. Antigen preparations obtained by immunoaffinity-absorption and analyzed by SDS-PAGE, immunoblotting on PVDF membranes and Amido Black staining appeared to contain ~20% E48 antigen and five major copurifying proteins, which have not been identified further as yet. For amino acid sequencing the E48 antigen band was excised from the PVDF membrane. The following amino acid sequence of the amino-terminal end was determined: LR(?)HVVTSNNK. As cysteines cannot be sequenced regularly, a putative C on position 3 was filled in since it was observed that a specific amino acid could not be detected on this position. After a few amino acids the signal decreased abruptly, usually an indication for amino acid modifications. The amino acid sequence was not found in the GENBANK and EMBL databases (searched by the FASTA program), but the sequence appeared to be similar to the mouse ThB antigen, a 15–20-kD protein on the surface of murine lymphocytes. The similarity found was as follows:

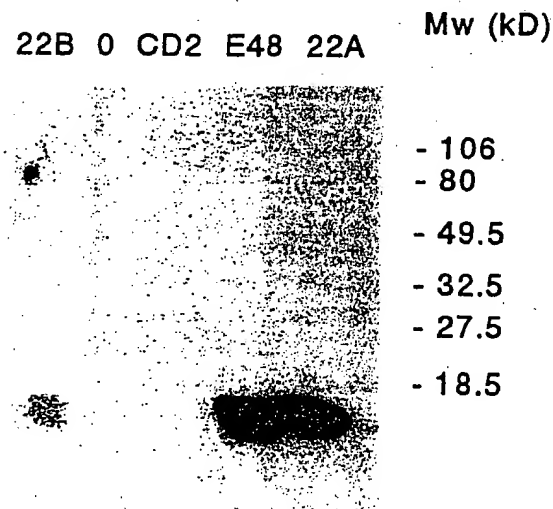
hE48	L	R	?	H	V	V	T	S	S	S	N	N	K
	*	*		*	*		*		*		*		*
mThB	L	R	C	H	V	C	T	N	S	A	N	C	K

Based on this sequence various sets of degenerated primers were synthesized: (a) sense: GN TGC/T CAC/T GTN GTN AC (E48), TCN TCN AAC/T AAC/T AA (E48), GN TGC/T CAC/T GTN TGC/T AC (ThB), and (b) antisense: A/GTT A/GCA NAG A/GTC NNN NNN A/GCA A/GCA (based upon the Ly-6 amino acid consensus sequence CCXXDLN: Gumléy et al., 1992). The primers were used in various methods of reverse transcriptase polymerase chain reactions (RT-PCR), hybridizations on Southern and Northern blots and PCR on commercially available keratinocyte cDNA libraries cloned in  $\lambda$  gt11 (Clontech; Westburg, Leusden, The Netherlands). Obtained PCR fragments were cloned in pUC19, and those containing both primers and with an insert length of at least 400 bp were partially sequenced. Only six clones contained an open reading frame. They were all unknown in the GenBank and EMBL databases, but none hybridized specifically with RNA isolated from squamous cell lines (data not shown).

### Molecular Cloning of the E48 Encoding cDNA

Simultaneously, expression cloning in eukaryotic vector/host systems was set up. CD2 cDNA cloned in the expression shuttle vector pCDM8 and anti-CD2 monoclonal antibody were used as a model system to optimize and simplify the transfection of COS cells, to enhance the detection and subsequent isolation of immunocytochemical stainable cells, and to optimize electrotransformation of *E. coli* MC1061/P3 (Rommens et al., 1991; Brakenhoff et al., 1994b). A cDNA library was generated from RNA of UM-SCC-22A, a HNSCC cell line with a high level of E48 antigen expression, in expression vector pCDM8, and

electrotransformed in *E. coli* MC1061/P3. A library with a complexity of  $7 \times 10^5$  independent colonies was generated and divided in nine fractions. Frozen glycerol cultures were prepared from these fractions and similarly DNA was isolated and transfected into  $2 \times 10^6$  COS cells. Immunocytochemical staining in situ revealed 80 positive clones. Plasmid DNA was isolated from positively stained cells scraped from the tissue culture dish by Hirt extraction, and retransformed in *E. coli* MC1061/P3 (Brakenhoff et al., 1994b). Plasmid DNA was isolated from the resulting colonies (~500 colonies/Hirt extraction) and retransfected. The CD2 cDNA clone (Seed and Aruffo, 1987) was used as a control for this established procedure, and was positive in all cases (Brakenhoff et al., 1994b). However, the E48 cDNA clone could not be isolated by this same method probably due to COS cell induced damage of the E48 cDNA clone (Brakenhoff et al., 1994b; Wijngaard et al., 1992 and references therein). By screening progressively smaller pools of bacterial colonies one of the positive clones could be enriched to 100% purity. Although this is a laborious method, it is completely reliable as the shuttling of plasmid DNA through COS cells is omitted: the transfection and immunocytochemical staining are used only to detect a positive pool of clones. We were able to clone the E48 antigen encoding cDNA in six steps. Transfection of the clone in COS cells and immunoblotting of the cell extracts proved that it encoded the correct antigen (Fig. 2). The clone was sequenced, the open reading frame determined and the location of a ribosome binding site and a signal peptide analyzed by computer calculations. Three putative cleavage sites for a signal peptide were found, of which one would result in a protein with the amino-terminal sequence LRCHVCTSSSNCK (Fig.



**Figure 2.** Immunoblotting of COS cells transfected with the E48 cDNA clone. COS cells were transiently transfected with no DNA (0), the CD2 cDNA clone in pCDM8 (CD2), and the E48 cDNA clone in pCDM8 (E48), and harvested 48 h posttransfection. Cell extracts of the transfected COS cells, and cell extracts of UM-SCC-22A (22A) and UM-SCC-22B (22B) as positive control, were run on a 12.5% SDS-PAGE gel and electroblotted to nitrocellulose. The blot was incubated with radiolabeled mAb E48, and exposed to Kodak X-AR 5 film for 1 wk using intensifying screens. The pattern of a molecular mass marker in kD (Bio-Rad) is indicated on the right.



3). As indicated earlier, microsequencing of the purified protein revealed the amino-terminal sequence LR(?) HV-VTSSSNK, indicating that this cleavage site is most likely correct. Not only the third amino acid appeared to be a cysteine but the sixth and the twelfth as well. Reevaluation of the sequencing chromatograms indicated that the signal of the 6th amino acid could indeed be the residual signal of the 5th valine (V), and that of the 12th amino acid the residual signal of the 11th asparagine (N).

The E48 cDNA sequence has an open reading frame of 128 amino acids from base 18-401, encoding a core protein with a molecular mass of 13,286 kD. In the amino acid sequence 2 theoretical phosphorylation sites (Thr20 and

Ser41), and 3 putative myristylation sites (Gly 75, 80 and 114, respectively) are present (Fig. 3).

The putative polyadenylation signal GATAAA of the E48 encoding cDNA is not according to the general consensus sequence AATAAA or ATTAAA (Proudfoot, 1989, 1991; Wahle and Keller, 1992). The activity of poly(A) polymerase on this signal sequence GATAAA has been measured in vitro by others, and appeared to be only 10% as compared to the consensus sequence (Wickens, 1990). To exclude that this nonconsensus sequence was due to reverse transcriptase infidelity, five additional clones were isolated from other fractions of the cDNA library by colony hybridization, and sequenced. The sequences revealed that all clones were of different length, but in all clones the GATAAA sequence appeared to be the most probable polyadenylation signal. The same sequence could be confirmed in a genomic clone (see also below). The use of polyadenylation signals not applying to the consensus rules are not specific for this antigen only. Similar irregular sequences have been observed for the CD59 gene as well, although in this case a consensus signal sequence was also present (Tone et al., 1992).

Database searching indicated a high sequence similarity of the human E48 antigen with the mouse ThB antigen, the family of the murine Ly-6 antigens, and human CD59 (LeClair et al., 1986; Davies et al., 1989; Gumley et al., 1992; MacNeil et al., 1993; Fleming et al., 1993). The ThB antigen and the family of Ly-6 antigens are all 15-20-kD membrane bound proteins expressed on lymphocytes, and anchored with a glycosyl-phosphatidylinositol (GPI) anchor to the membrane. The molecules are thought to be involved in signal transduction, but the corresponding ligands are unknown. The human CD59 molecule is expressed on erythrocytes and is involved in the inhibition of autologous complement attack (Fig. 4).

```

10      20      30      40      50      60
GAATTCGACATCAGAGATGAGGACAGCATTGCTGCTCCTTGCCAGCCCTGGCTGTGGCTA
70      80      90      100     110     120
T G P A L T L R C H V C T S S S N C K H
CAGGGCCAGCCCTTACCTGCGGTGCGACGCTCCAGCACTGCAAGCAATT

130     140     150     160     170     180
V V C P A S S R F C K T T N T V E P L
CTGTGCTGCTGCCCGCCAGCTCTCGCTTTCGAAGACCAACACAGTGGAGCCTCTGA

190     200     210     220     230     240
R G N L V K K D C A E S C T P S Y T L Q
GGGGGAATCTGGTGAAGAAGGACTGTGCGGAGCTGTCACACCCAGCTACACCCCTGCAAG

250     260     270     280     290     300
G Q V S S G T S S T Q C C Q E D L C N E
GCCAGTCCAGCAGCGGCCAGGCTCCACCCAGTGTGCGGAGGAGGAGCTGTGCAATGAGA

310     320     330     340     350     360
K L H N A A P T R T A L A H S A L S L G
AGCTGCACACGCTGCACCCACCCGACCGCCCTGCGCCACAGTGCCTCAGCCTGGGGC

370     380     390     400     410     420
L A L S L L A V I L A P S L ***
TGGCCCTGAGCCTCTGGCGCTCATCTTAGCCCCCAGCCTGTGACCTTCCCCCAGGGAA

430     440     450     460     470     480
GGCCCCCTCATGCTTTCTTCTCTCTGCGGATTCCACACCTCTCTTCCCCAGCCGG

490     500     510     520     530     540
CAACGGGGTGGCAGGAGCCCGAGGCTGAGGGCTTCCCGAAAGTCTGGGACAGGTCCA

550     560     570     580     590     600
GGTGGGCATGGAATGCTGATGACTTGGAGCAGGCCCCACAGACCCACAGAGGATGAAGC

610     620     630     640     650     660
CACCACACAGAGGATGAGCAGCCCGAGCTGCATGGAAGTGGAGGACAGAAGCCCTGTGGA

670     680     690     700     710     720
TCCCGGATTTCACACTCTTCTGTTTGTGTCGCTTATTTGTACTCAATCTCTACA

730     740     750     760     770
TGGAGATAAATGATTAAACCAAAAAAAAAAATCTAGAGTCGGAATTC

```

Figure 3. Sequence of the E48 encoding cDNA. The sequence of the E48 encoding cDNA clone, isolated by expression cloning in COS cells, is given in capitals. The underlined amino acid sequences were determined by amino acid sequencing of the purified antigen except for the cysteines indicated with C\*. The putative polyadenylation sequence is indicated in bold and italicized. The stop codon is indicated with asterisks. The XbaI site at the 3' end as well as the EcoRI sites at the 5' and 3' ends of the cDNA are derived from the oligo(dT) primer-adaptor and BstXI/EcoRI adaptors used for the construction of the cDNA library, respectively. The sequence is accessible in the EMBL database as X82693 HSE48.

hE48	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34
mThB	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34
mLy-6A.2/E.1	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34
mLy-6F.1	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34
mLy-6G.1	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34
mLy-6C.2	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34
mTSA-1	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34
hCD59	L	R	C	H	V	C	T	S	S	S	N	C	K	H	S	V	C	P	A	S	S	R	F	C	K	T	T	N	T	V	E	P	L	34

hE48	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73
mThB	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73
mLy-6A.2/E.1	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73
mLy-6F.1	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73
mLy-6G.1	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73
mLy-6C.2	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73
mTSA-1	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73
hCD59	R	G	N	L	V	K	K	D	C	A	E	S	C	T	P	S	Y	T	L	Q	73

Figure 4. Homology of the human E48 antigen with the members of the murine Ly-6 antigen family and the human CD59 antigen. Sequence similarity of the members of the murine Ly-6 family and the human CD59 protein with the human E48 antigen was determined by the computer program Clustal, PCgene, Intelligenetics. Conserved residues are boxed, only the protein sequences as expressed on the cell membrane are shown. The gaps indicated with asterisks were additionally introduced to align the 7th conserved cysteine residue. Identical residues of the mouse ThB and the human E48 antigen are indicated in bold. Sequences were taken from LeClair et al. (1986), Palfree et al. (1988), Davies et al. (1989), Gumley et al. (1992), Fleming et al. (1993), and MacNeil et al. (1993).

### The E48 Antigen Is a GPI-anchored Membrane Protein

The homology with the Ly-6 family, proteins attached to the membrane with a GPI anchor suggested that the E48 antigen might be attached similarly. The presence of a GPI-anchor was measured by PI-specific phospholipase-C treatment of antigen positive cells, a method often used to demonstrate the GPI-anchoring of a surface protein (Cross, 1990). Extracts of PI-specific phospholipase-C-treated COS cells transfected with the E48 encoding cDNA were analyzed by immunoblotting (not shown), and PI-specific phospholipase-C-treated UM-SSC-22A cells were analyzed by immunocytochemistry (not shown) and FACS. The flow cytometry analysis is shown in Fig. 5. UM-SCC-22A cells treated with anion free buffer and incubated with E48 antibody showed a high fluorescence, whereas after PI-specific phospholipase-C treatment in anion free buffer and subsequent E48 incubation, the UM-SCC-22A cells showed a very low level of fluorescence. Similar results were obtained in a control assay of the GPI-anchored MOv18 antigen (folate-binding protein) overexpressed on KB cells (Luhrs and Slomiany, 1989; Coney et al., 1991; data not shown). These data clearly indicated that the E48 antigen is released from the cell surface by PI-specific phospholipase-C treatment, demonstrating that the protein is GPI-anchored to the plasma membrane.

### Expression Pattern and Chromosomal Localization of the E48 Encoding Gene

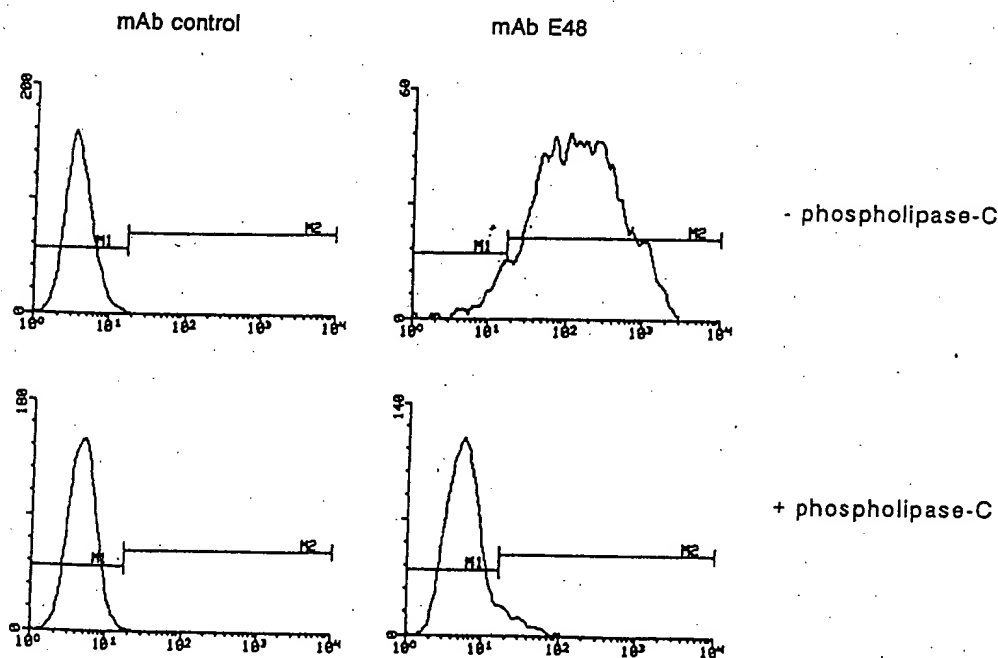
Northern blotting experiments revealed that expression of the E48 gene is confined to squamous cell carcinoma cell lines and squamous epithelia (Fig. 6 B, Table I). The transcript is ~0.8 kb in length. Splice variants could not be de-

tected by Northern blotting, not even after overexposure of the autoradiogram. There appears to be a direct relationship between the antigen expression as detected by immunoblotting and immunocytochemistry (20% of the UM-SCC-22B cells, and 100% of the UM-SCC-22A cells are positive), and the relative amount of E48 mRNA in these cells.

Southern blotting experiments indicated that the E48 antigen is encoded by a single copy gene (Fig. 6 A). Subsequent analysis of a panel of 24 somatic cell hybrids revealed significant segregation with human chromosome 8, with a single exception probably due to chromosomal rearrangement after cell fusion (Table II). To obtain a regional localization of the gene on chromosome 8, a clone containing the E48 gene was isolated from a genomic DNA library in lambda EMBL3 (Brakenhoff et al., 1994a) and used for *in situ* hybridization using biotin-labeled probes. This analysis clearly showed two regions of hybridization, one on chromosome 8q24-qter and one on chromosome 15q22 (Fig. 7). Since the somatic hybrid panel did not show any concordance with chromosome 15, we concluded that the E48 encoding gene is located on chromosome 8, in the 8q24-ter region. The hybridization of the genomic probe (~20 kb in length) to chromosome 15 is most likely due to cross-hybridization with an as yet unidentified pseudogene or with low copy repetitive sequences, as was indicated by Southern blot hybridization (not shown). Restriction mapping and sequencing of the gene are in progress.

### Characterization of the Putative Mouse Homologue, the ThB Antigen

An unexpected observation was the high sequence similarity with the murine ThB antigen, a GPI-anchored mole-



**Figure 5.** Flow cytometry analysis of E48 antigen expression on UM-SCC-22A cells with and without PI-specific phospholipase-C treatment. UM-SCC-22A tumor cells were trypsinized and incubated in anion free buffer with (+) or without (-) PI-specific phospholipase-C, followed by incubation with mAb E48 or an isotype-matched control antibody (mAb control), and goat anti-mouse FITC immunoglobulin conjugate. The cells were analyzed by flow cytometry, and the corresponding histograms are shown. On the X-axis, arbitrary units of fluorescence are indicated, and on the y-axis the number of cells counted. The M1/M2 gates were adjusted to 0.5% positive cells with the negative control antibody. Note that the E48 antigen is released from the plasma membrane of the UM-SCC-22A cells by PI-specific phospholipase-C treatment.

A

EcoRI BamHI HindIII

23.1 -

9.4 -

6.7 -

4.3 -

2.3 -

2.0 -

B

COS MOP8 22A 22B KER

(kb)

- 9.5

- 7.5

- 4.4

- 2.4

- 1.4

- 0.24

cule expressed on lymphocytes (Fig. 4). It had already been described earlier that two allelotypes of the ThB antigen exist: ThB<sup>h</sup> (C57Bl/6 mice) having a high level of expression, and ThB<sup>l</sup> (BALB/c mice) having a low level of expression in lymphocytes (Gumley et al., 1992). The ex-

Table I. Expression of the E48 Gene in Tumor Cell Lines

Cell line	Origin	Positive
UM-SCC-22A	squamous cell carcinoma	++
UM-SCC-22B	squamous cell carcinoma	+
OVCAR-3	adenocarcinoma ovary	-
WiDr 29	adenocarcinoma colon	-
A431	epidermoid carcinoma vulva	+
LNCAP	adenocarcinoma prostate	-
Tera-1	teratocarcinoma	-
MCF-7	adenocarcinoma breast	-
DOHH2	B-cell lymphoma	-
NB-1	neuroblastoma	-
U715	B-cell lymphoma	-
Raji	Burkitt lymphoma	-
Hela	epitheloid carcinoma cervix	-
SiHa	squamous carcinoma cervix	+
PC112	pheochromocytoma	-
<b>Primary cells/tissues</b>		
keratinocytes	oral cavity	+
peripheral blood mononuclear cells		-
adrenal gland		-

In the first column the codes of the human cell lines and tissues are given; in the second, the type of tumor and the tissue of origin, and in the third, the presence or absence of E48 mRNA as determined by Northern blotting.

pression pattern of ThB in squamous tissue, i.e., skin, was not determined. We therefore analyzed the ThB expression in various tissues of mice of the ThB<sup>h</sup> (C57Bl/6) and ThB<sup>l</sup> (BALB/c) allelotypes. RNA was isolated, Northern blotted, and hybridized with the ThB cDNA as a probe (kindly provided by Dr. M. Sandrin). In brain, skeletal muscle, colon, heart, kidney, and liver no ThB transcripts could be detected, and in spleen, as expected, only a very low level for the ThB<sup>l</sup> (BALB/c) and a relatively high level for the ThB<sup>h</sup> (C57Bl/6) allelotypes. However, in tongue, tail skin, and ear skin a very high level of ThB RNA could be detected. As shown in Fig. 8, the expression of ThB in keratinocytes is not only 10–100 times higher as compared to the spleen of the ThB<sup>h</sup> allelotype, but also independent of the mouse strain. Only the expression in spleen (lymphocytes) is strain dependent, being high in C57Bl/6 and low in BALB/c. This expression pattern strongly suggests that the ThB antigen is most likely of critical importance on murine keratinocytes and less on lymphocytes. The function of the ThB antigen on murine keratinocytes will most likely be similar with that of the putative human homologue, the E48 antigen, on human squamous cells. The role of the ThB antigen on lymphocytes remains as yet unclear, although involvement with signal transduction has been suggested (Gumley et al., 1992). As yet, we never observed E48 expression on lymphocytes in man.

#### Functional Characterization of the E48 Antigen

In previous papers the function of the E48 antigen was linked to desmosomal cell adhesion. The molecule could

Figure 6. Southern blot and Northern blot hybridized with the E48 cDNA. (A) 10 µg human genomic DNA was digested with EcoRI, BamHI, and HindIII, respectively, run on a 0.7% agarose gel, Southern blotted and hybridized with the E48 cDNA as a probe under moderate stringent conditions. After hybridization the blot was exposed to Kodak X-AR 5 film for 64 h using intensifying screens. The fragment lengths in kilobases of lambda DNA cut with HindIII are indicated on the left. (B) 20 µg of total RNA isolated from COS-7, MOP-8 UM-SCC-22B cells and cultured primary keratinocytes were run on a 1% agarose/formaldehyde gel, Northern blotted and hybridized with the E48 cDNA as a probe. The blot was exposed to Kodak X-AR 5 film for 24 h using intensifying screens. The length in kilobases of an RNA standard (BRL, Gibco Life Technologies) is indicated on the right.



**Table II. Segregation of Human Chromosomes and/or Chromosome-Specific Markers and the E48 cDNA in Rodent/Human Somatic Cell Hybrids**

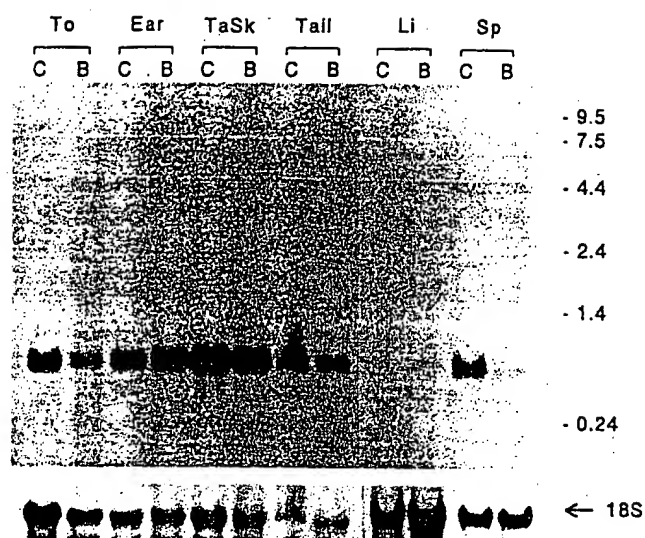
Chromosome	E48/chromosome; No. of clones				Discordancy %
	+/+	+/-	-/+	-/-	
1	6	5	5	8	42
2	4	7	1	12	33
3	5	6	3	10	38
4	9	2	3	10	21
5	6	5	5	8	42
6	7	4	2	11	25
7	7	4	6	7	42
8	11	0	1	12	4
9	5	6	3	10	38
10	8	3	4	9	29
11	5	6	6	7	50
12	8	3	5	8	33
13	8	3	3	10	25
14	7	4	5	8	38
15	4	7	8	5	63
16	10	1	6	7	29
17	10	1	9	4	42
18	7	4	6	7	42
19	10	1	5	8	25
20	9	2	3	10	21
21	6	5	5	8	42
22	8	3	6	7	38
X	9	2	4	9	25

Southern blots with DNA isolated from human/rodent cell hybrids were hybridized with the E48 cDNA as probe, and the categories of discordants counted separately.

be detected in the desmosomal midline by immunoelectronmicroscopy and was shown to influence cell adhesion in in vitro model systems (Schrijvers et al., 1991). Although the homology of the E48 sequence with the mouse



**Figure 7. Regional localization of the E48 gene on human chromosome 8q24-qter.** Metaphase spreads of human chromosomes were hybridized in situ with a biotin-labeled EMBL3 lambda clone containing the E48 gene. The chromosomes were counterstained and analyzed as described by Suijkerbuijk et al. (1992). Note the white spots in the centromeric region (upper right) of chromosome 15, and in the telomeric region (8q24-qter) of chromosome 8 (middle right).

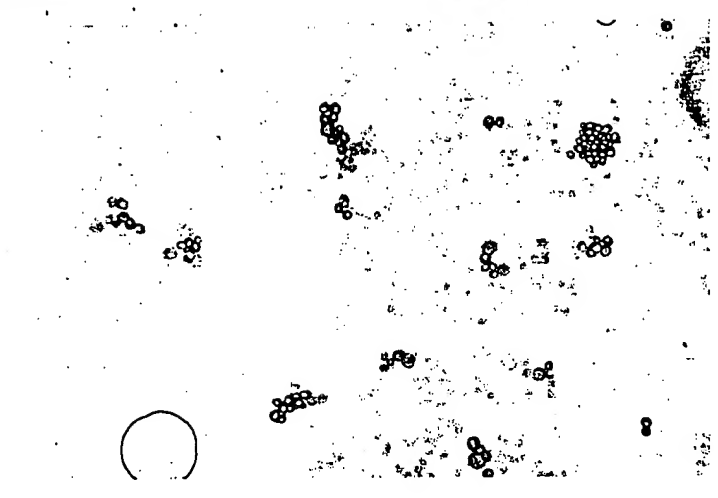


**Figure 8. Expression of the ThB antigen in squamous epithelia of the mouse.** RNA was isolated from C57Bl/6 (C) and BALB/c (B) tongue (To), ear, separated epidermis of tail skin (TaSk), tail skin (Tail), liver (Li), and spleen (Sp). Approximately 10 µg (Tail, 3 µg; Ear, 7 µg) was run on a 1% agarose/formaldehyde gel, blotted to genescreen plus and hybridized with the ThB cDNA as probe under moderate stringent conditions. The blot was exposed for 64 h (To, Ear, TaSk, Tail) or 2 wk (Li, Sp) to Kodak X-AR 5 film using intensifying screens. Note the large difference in the level of ThB transcripts in spleen RNA (Sp) between the ThB<sup>h</sup> (C57Bl/6, lane C) and ThB<sup>l</sup> (BALB/c, lane B) mouse strains, whereas the differences in RNA level between these strains in squamous tissues (To, Ear, TaSk, Tail) is negligible. The blot was subsequently hybridized with an 18S rRNA probe, and exposed for 2 h to Kodak X-AR 5 film. The hybridizing bands are indicated at the lower panel.

Ly-6 family suggested otherwise, the role of the E48 antigen in cell-cell adhesion was clearly demonstrated when the encoding cDNA in expression vector pCDM8 was transiently transfected into MOP-8 cells. After transfection the cells were cytopinned on microscopical glass slides and immunocytochemically stained with the E48 antibody. As shown in Fig. 9, all E48 positive cells aggregate, whereas the E48 negative cells remain dispersed, indicating that the molecule is involved in MOP-8 cell-cell interaction. The experiment was repeated with and without the addition of mAb E48 or an isotype-matched control mAb in the culture medium at a final concentration of 100 µg/ml. Although mAb E48 at the same concentration was able to inhibit cell clustering of UM-SCC-22B cells in collagen gels (Schrijvers et al., 1991), the aggregation of the transiently transfected MOP-8 cells could not be inhibited. Cells transfected with the WC1 antigen did not show this kind of aggregation (Wijngaard et al., 1992; not shown).

## Discussion

The E48 antigen is a target molecule for antibody-based immunotherapy, and to envision the results of high dose antibody treatment in patients with squamous cell carcinoma, and to evaluate the potential role of the antigen in carcinogenesis and tumor progression, we decided to clone the cDNA as well as the gene to unravel its function. The



**Figure 9.** Functional characterization of the E48 region. MOP-8 cells were transiently transfected with the E48 cDNA in pCDM8. They were removed from the culture flask after 48 h by mild trypsinization, and spun on microscopical glass slides, coated with 0.1% poly-L-lysine, and immunocytochemically stained by mAb E48 and APAAP. Note the striking clustering of transfected and E48 expressing cells, while the nontransfected cells are dispersed.

cDNA encoding the E48 antigen contained an open reading frame of 401 nucleotides encoding a polypeptide of 13,286 kD. Scanning the E48 polypeptide for posttranslational modification sequences revealed two putative protein kinase C phosphorylation sites and three putative N-myristylation sites. Comparison with a similar analysis on the ThB polypeptide, the putative mouse homologue (see below), revealed that only the N-myristylation site on the glycine at position 80 is conserved. The actual use of these sites should be established by further research.

Surprisingly the E48 antigen appeared to have a very high sequence similarity with the ThB antigen, of which the cDNA was cloned from a C57/Bl6 mouse spleen cDNA library (Gumley et al., 1992). This antigen belongs to the larger Ly-6 family of 13–15 kD surface proteins, GPI-anchored to the membrane and initially identified on murine lymphocytes (See also Fig. 4). The E48 antigen is the first member of the human family except for perhaps the CD59 antigen. The homology with the Ly-6 family is not only confined to the sequence similarity and the comparable expression pattern (see below), but also to the chromosomal localization of the genes. The E48 gene was assigned to human chromosome 8 in the q24-qter region, where also the myc gene has been mapped. The various members of the mouse Ly-6 family have all been mapped to mouse chromosome 15, in proximity of the myc gene (Hogarth et al., 1987; Gumley et al., 1992; MacNeil et al., 1993; Fleming et al., 1993). These observations stress the putative homology of the E48 antigen with the mouse ThB antigen, since this part of human chromosome 8 is syntenic with mouse chromosome 15 (Donis-Keller and Buckle, 1991; Genome Database). It should be noted that the other member of the human family of the Ly-6 like GPI-anchored antigens, CD59, is located on chromosome 11p13 (Forsberg et al., 1989; Bickmore et al., 1993). In addition, the sequence similarity of the coding regions of the CD59 and E48 genes are below 50% suggesting that these

genes, if they have arisen from a common ancestor, must have been separated long ago in evolution.

It seems obvious that, as in mice, a family of Ly-6 antigens will exist in man, most likely clustered at the chromosomal region 8q24-qter. Although several members of the murine Ly-6 gene family were cloned already in 1987, the identification of the human family has been unsuccessful, probably due to similar technical difficulties as we encountered in the molecular cloning of the E48 encoding cDNA. At the time we at first obtained amino acid sequences of the E48 antigen, we synthesized degenerated oligonucleotides for PCR and screening of cDNA libraries based on the sequence LRCHVVT. As the homology with the ThB antigen could already be detected on the protein level (being LRCHVCT), we also generated primers based upon the ThB sequence, as well as the consensus sequence of most Ly-6 members CCXXDLN, the sequence attached to the GPI-anchor. Although it was confirmed later that the amino-terminal sequence and the GPI-linked consensus sequence CCXXDLN of the ThB antigen were similar to those of the E48 antigen, RT-PCR with primers based on these amino acid sequences did not result in the amplification of any specific fragment. This negative result is probably due to the (N)<sub>6</sub> nucleotide sequence in the antisense primer.

What is more, cross-hybridization, which is often used to clone cDNAs and genes from other species, is also difficult. Only when a very small fragment of the E48 cDNA encompassing the coding sequences of the processed protein (a cloned fragment amplified between the ThB-sense- and Ly-6 consensus-antisense-primers: see Materials and Methods), was used as a probe, cross-hybridization without a high background could be detected on Southern blots of genomic murine DNA. Ly-6 genes within one species cross-hybridize (Kamiura et al., 1992; Fleming et al., 1993) much more specifically, and the molecular cloning of other members of the putative human family should be facilitated by the identification of the putative human ThB gene homologue, and its chromosomal localization. From the mouse locus it has been shown that all Ly-6E.1 hybridizing sequences could be found in a single 650-kb fragment (Kamiura et al., 1992). Isolation of YAC clones from a similar region of chromosome 8 using the E48 cDNA as a probe should most likely enable the molecular cloning of other members by subsequent cross-hybridization or even PCR strategies.

A number of hereditary diseases have been mapped to chromosome 8q24-qter, such as the Langer-Giedion syndrome, the brachio-otorhinolaryngeal syndrome, the trichorhinolaryngeal syndrome I and the epidermolysis bullosa simplex (OGNA). Some of the clinical features, especially those related to the skin and hair phenotypes might be explained by the deletion of the E48 gene in these genetic diseases (Donis-Keller and Buckle, 1991; MIM [mendelian inheritance of man] database). Particularly when a larger Ly-6 gene family is present at this locus, various hereditary genetic disorders could be due to (partial) deletion of this chromosomal region. Experimental proof of the involvement of the other putative human Ly-6 antigens in these syndromes could only be evaluated in gene targeting experiments in mice.

As indicated by Northern blotting and hybridization, the

expression of the E48 antigen is confined to squamous tissues. In mice the putative homologue, ThB, is expressed in lymphocytes as well, although at a much lower level. Moreover, on lymphocytes the expression level is mouse strain dependent, and two phenotypes, ThB<sup>h</sup> and ThB<sup>l</sup> have been described (Gumley et al., 1992). We show here that the expression of the ThB antigen appears to be much higher in squamous tissues, and, what is more, independent of the mouse strain. The variation of ThB expression in lymphocytes between the two mouse strains had already been explained by a possible difference in a transcription factor recognition sequence in the promoter/enhancer sequence of the two alleles ThB<sup>h</sup> and ThB<sup>l</sup>, resulting in different levels of ThB gene expression (Gumley et al., 1994). The promoter/enhancer sequence of the human E48 gene has probably lost the capacity to bind lymphocyte transcription factors, and is therefore not expressed in cells of the lymphocyte lineage.

The ThB/E48 antigen is not the only Ly-6 antigen expressed on keratinocytes. The Ly-6A.2 antigen has also been detected on mouse keratinocytes (Hogenesch et al., 1993), and, what is more, other members of the Ly-6 family are expressed in various other tissues and organs (Cray et al., 1900; Blake et al., 1993; Fleming et al., 1993; Gordon et al., 1993; Hogenesch et al., 1993). We have investigated the expression of CD59 in human keratinocytes by Northern blotting and immunohistochemistry, but we found only a very low expression level in keratinocytes (data not shown). These and other published data will make it necessary to consider renaming the antigens. hThB: human Thymocyte and B-cell antigen, is not a very logical name for the E48 antigen, when it is exclusively expressed in keratinocytes. We would therefore propose to rename the Ly-6 antigens into CRG proteins: Cysteine Rich GPI-anchored proteins, and, for the time being, add part of the original name. When the genes encoding these proteins have all been cloned from one species, a definite nomenclature could be agreed, for instance in the direction of transcription, or, if the directions of transcription are different, in the direction from centromere to telomere. A similar consensus has been reached in the nomenclature of the  $\gamma$ -crystallin gene family in various mammalian species (Aarts et al., 1988). A prefix to the name could be added to indicate the species from which the antigen has been identified. mCRG-A2/E1, mCRG-C2, mCRG-F2, mCRG-G2 (formerly Ly-6A.2/E.1, Ly-6C.2, Ly-6F.2, Ly-6G.2, respectively; it should be noted that Ly-6A.2 and E.1 are most likely alleles of one gene), mCRG-TSA (formerly mouse TSA antigen) mCRG-ThB (formerly the ThB antigen), hCRG-E48 (formerly, the human E48 antigen), hCRG-CD59 (formerly the CD59 antigen, the first human gene identified but on a different locus). Although we realize that changing names is a difficult and annoying process and should only be performed when there is a consensus in the field (Palfree, 1991), we think that the trivial name "E48 antigen" should be adapted as soon as possible.

The function of the human E48 antigen and most likely of the mouse ThB antigen in skin, is apparently different from the reported function of the Ly-6 family of antigens in murine lymphocytes. The members of this larger family of GPI-anchored molecules have been shown to be in-

volved in signal transduction. The murine Ly-6A.2 antigen as well as the human CD59 antigen interact with tyrosine kinases of the src family and the ThB antigen probably to a tyrosine phosphatase, all involved in protein phosphorylation cascades (Stefanova et al., 1991; Gumley et al., 1992; Shenoyscaria et al., 1992; Bohuslav et al., 1993; Shenoyscaria et al., 1993). Cross-linking of the Ly-6 molecules by antibodies resulted in lymphocyte activation and proliferation (Malek et al., 1986; Havran et al., 1988; Rock et al., 1989). To test the hypothesis that cross-linking of the E48 antigen by its corresponding antibody might cause similar effects, we have measured the effect of the antibody binding on the growth behavior of keratinocytes and squamous carcinoma cells in culture by standard assays. Although control antibody K984 which has a direct influence on cell proliferation showed considerable effects, the E48 antibody had no effect (Schrijvers et al., 1992; data not shown). However, it cannot be excluded that only cross-linking of specific epitopes on these molecules cause signal transduction, that these effects can be determined in (murine) lymphocytes only, or that the culture conditions of keratinocytes are not permissive for these effects.

In model assays it has been demonstrated that the E48 antigen is involved in cell-cell adhesion. The role of a GPI-anchored Ly-6 antigen in adhesion processes is novel. GPI-anchored proteins are thought to be involved in potocytosis: rapid transport processes without formation of endosomes, or signal transduction (Brown and Rose, 1992; Anderson, 1993; Zurzolo et al., 1993; Zurzolo et al., 1994). GPI-anchored proteins can form microdomains on the membrane localized in caveolae, and characterized by the coat protein caveolin, although the existence of these domain formations is still controversial (Rothberg et al., 1992; Mayor et al., 1994). Involvement of GPI-anchored proteins in cell-cell adhesion has been described earlier: the human carcinoembryonic antigen (CEA) is a GPI-anchored adhesion molecule (Eidelman et al., 1993). We have shown that the E48 antigen, transfected into MOP-8 fibroblasts induces cell aggregation. It was a little surprising that the aggregation could not be blocked by the specific antibody in this system as it has clearly been demonstrated that the cell adhesion of UM-SCC 22B cells could be perturbed by E48 antibody (Schrijvers et al., 1991). This observation could be explained by the different use of the antigen in the various cell types, and its involvement in adhesion processes which necessitate different associated proteins in distinct cells. Experiments with stable transfected cell lines in combination with coimmunoprecipitations should elucidate the precise function of the molecule and the involvement of associated proteins.

The indicated influence on cell aggregation is consistent with the postulated similarity of the E48 antigen with the 20-kD protein identified in desmosome-enriched preparations of bovine muzzle, formerly termed desmoglein III/dg 4 (See Introduction; Schwarz et al., 1990; Schrijvers et al., 1991). To support this idea we have prepared a desmosome-enriched fraction from human xenografts and showed that the E48 antigen is coenriched in this isolation. It should be noted, however, that this 20-kD protein had been reported to be glycosylated (Gorbsky and Steinberg, 1981), whereas the E48 sequence did not reveal any glycosylation sites. These conflicting findings can be explained by the chemi-

cal method used by Gorbisky and Steinberg to detect carbohydrates on the desmosomal components. They described the isolation of desmosomes from bovine muzzle, and the detection of carbohydrates by periodate/dansyl-hydrazine treatment. The reported weak signal of the 20-kD protein could, in view of the data presented in this paper, be explained by the detection of the inositol and the other core sugar residues in the GPI-anchor, all reactive with periodate (Cross, 1990; Fournie et al., 1991). These data demonstrate that the E48 antigen and the 20-kD protein formerly termed desmoglein III/dg 4 are most likely identical.

It is tempting to speculate that the role of the Ly-6 molecules in general is not directly related to the cell proliferation, but only indirectly. It has firmly been established that GPI-anchored molecules are concentrated in specialized membrane domains, and the general function of the small cysteine-rich Ly-6 molecules could be the formation of these specialized membrane domains together with caveolin (Ferguson, 1988; Rothberg et al., 1992; Sargiacomo et al., 1993; Garcia et al., 1993). The different components of the particular domain could explain the different functions: on T-lymphocytes signal transduction, on MDCK and Caco-2 cells transport, and on keratinocytes cell-cell adhesion. It is obvious that this hypothesis should be proved experimentally, but the observation that CD59 on erythrocytes functions in protection against autologous complement attack, and in T-lymphocytes functions in signal transduction, comparable to the mouse Ly-6A.2 molecules, strongly supports the theory (Korty et al., 1993).

Summarized, with the molecular cloning of the cDNA encoding the first member of the human Ly-6 family, a tool for the molecular cloning of cDNAs encoding other members of the human family is available, enabling the functional characterization of these GPI-anchored antigens in the various cells and tissues of man.

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